The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation

(viral transactivator/transcriptional elongation/elongation factor)

MARK B. FEINBERG*, DAVID BALTIMORE*†, AND ALAN D. FRANKEL*‡

*Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142; and †The Rockefeller University, 1230 York Avenue, New York, NY 10021

ABSTRACT   The mechanism of Tat transactivation was studied by treating cell lines containing Tat-defective viruses with purified Tat protein. These cell lines constitutively produce very low levels of virus in the absence of Tat, as measured by p24 antigen levels. Virus production can be increased >30,000-fold by adding exogenous Tat. Tat addition increases mRNA levels early in the viral life cycle, and Tat is required for Rev function to become evident. There is no evidence for a translational effect of Tat. Nuclear run-on experiments show that the increase in mRNA levels is due to an increased efficiency of elongation of nascent transcripts. These results suggest that Tat may be a gene-specific elongation factor.

The Tat protein from human immunodeficiency virus type 1 (HIV-1) is a potent viral transactivator that is essential for viral replication. The precise mechanism of transactivation has been unclear. Several studies have shown that Tat increases the rate of transcription from the HIV long terminal repeat (LTR) (1–7), and some have suggested that Tat also increases translational efficiency (2–4, 8–10). Whether the transcriptional effect results from an increased rate of initiation or elongation has not yet been clearly determined. An elongation mechanism is supported by steady-state RNA analyses (11, 12), nuclear run-on experiments (7), and recent experiments using an *in vitro* transactivation system (13) and is consistent with the observation that Tat acts on nascent RNA transcripts (14).

Transactivation by Tat requires a region near the start of transcription in the viral LTR called the transacting-responsive (TAR) element (15). TAR RNA forms a stable stem-loop structure (4); Tat binds to a 3-nucleotide bulge in TAR (16–18), whereas cellular factors bind to a 6-nucleotide loop (19–21). Tat RNA-binding is important for transactivation both *in vivo* (16, 18) and *in vitro* (13), whereas the role of cellular factors remains to be determined.

Most studies to date have examined the mechanism of Tat transactivation by using transient assays and heterologous reporter systems. Here we describe the mechanism of action of Tat using intact HIV-1. We have taken advantage of the fact that purified Tat protein can be taken up by cells, enter the nucleus, and transactivate the HIV promoter (22, 23). By complementing Tat-defective viruses with purified protein, we have been able to precisely follow the action of Tat during the viral life cycle. We show that HIV-1 gene expression is temporally regulated and that Tat transactivation precedes Rev function. Analysis of RNA and protein expression shows that most, or all, of the Tat effect is transcriptional and that the increase in viral mRNAs results primarily from increased efficiency of elongation. Our results present a simple view of Tat function in HIV-1 and suggest that control of transcrip-

tional elongation may be an important mechanism for regulating cellular gene expression.

MATERIALS AND METHODS

Viruses and Cells. Wild-type HIV-1 particles were generated by transfection of the proviral plasmid R7/3, a derivative of sp65 HXBgpt (8). R7/Neo, R7/pNeo (−), and R7/pNeo (+) viruses are described in Fig. 1. CEM and H938 (25) cells were grown in RPMI 1640 medium/10% fetal bovine serum, and HeLa cells were grown in Dulbecco's modified Eagle's medium/10% fetal bovine serum.

Transfections and Infections. CEM cells were electroporated with 20 μg of supercoiled plasmid DNA per 10^7 cells, and HeLa cells were electroporated by using linearized plasmid DNAs. Tat-mutant viruses were generated by treating 10^7 transfected cells with purified Tat protein (22, 26) at 10 μg/ml. After 24 hr, cells were pelleted, and the supernatant was used to infect fresh CEM cells. Stable CEM cell lines were selected in G418 at 1 mg/ml and cloned by limiting dilution. Transfected HeLa cells were selected in G418 at 500 μg/ml.

Tat Complementation Assays. CEM or H938 cells (2.5 × 10^7) were treated with purified Tat protein, residues 1–72 (22, 27) at 10 μg/ml in 1 ml of medium containing protamine sulfate at 100 μg/ml. Protamine significantly enhances uptake and transactivation by exogenous Tat (D. A. Mann and A.D.F., unpublished work). Cells were incubated at 37°C for 15 min and then diluted to 10^6 cells per ml. HeLa cells were scrape-loaded with 10 μg of Tat in 2 ml of medium containing 100 μM chloroquine (27). Cells were lysed in 50 mM Tris·HCl, pH 8.0/150 mM NaCl/phenylmethylsulfonyl fluoride at 100 μg/ml/1% Nonidet P-40 and centrifuged to pellet nuclei. Protein concentrations were measured by the bicinchonic acid method (Pierce). HIV-1 viral core antigen (p24) and neomycin phosphotransferase were quantitated by ELISA (DuPont/NEN and 5 Prime → 3 Prime, Inc., respectively), and chloramphenicol acetyltransferase (CAT) assays were done as described (27).

RNA Analysis. Total RNA was isolated by the hot acidic phenol method (28) for RNase protection experiments and by guanidinium isothiocyanate/CsCl gradient centrifugation (29) for Northern (RNA) blots. ^{32}P-labeled HIV-1-specific RNA probes were prepared by *in vitro* transcription of an EcoRV-linearized plasmid containing the EcoRV (−120) to HindIII (+80) fragment of the viral LTR and were purified on Sephadex G-50 spin columns. RNase protection experiments were done as described (30). Twenty micrograms of cellular RNA was hybridized overnight with 5 × 10^5 cpm of the RNA probe at 38°C in 40 μl of 80% (vol/vol) formamide/40 mM Pipes, pH 6.7/200 mM NaCl/1 mM EDTA. Single-stranded

Abbreviations: LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; TAR, transacting-responsive element.
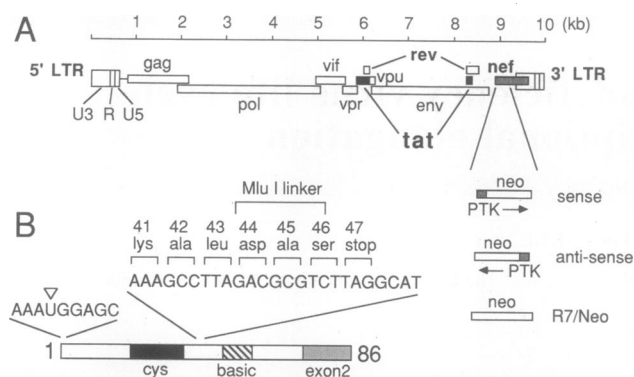‡To whom reprint requests should be addressed.

FIG. 1. Map of Tat-defective neo⁺ viruses. (A) The *neo* gene was cloned into the *nef* region (between nucleotides +8375 and +8630) of the HIV-1 genome under control of the polyoma enhancer/thymidine kinase (PTK) promoter cassette (1.1 kb from pMCIneo; ref. 24) in both the sense [R7/pNeo (−)] and antisense [R7/pNeo (+)] orientations. A promoterless *neo* gene was cloned at the same *nef* position, and its expression was driven by the 5′ HIV LTR (R7/Neo). (B) Tat mutations were introduced by deleting the second nucleotide (U; open triangle) in the *tat* initiation codon (4) and by inserting a synthetic *Mlu* I linker into the *Bsu*36I site of R7/3 at amino acid 43.

RNA was digested with RNase A at 10 μg/ml and RNase T1 at 45 units per ml in 400 μl of 10 mM Tris·HCl, pH 7.5/300 mM NaCl/5 mM EDTA for 1 hr at room temperature. Protected fragments were electrophoresed on 6% polyacrylamide-7 M urea sequencing gels and quantitated with a Betascope 603 (Betagen, Waltham, MA). For Northern blots, 20 μg of total cellular RNA was fractionated on 1.2% agarose-formaldehyde gels and transferred and hybridized as described (30).

Nuclear Run-on Transcription Assays. Nuclei were prepared (31), and nuclear run-on assays were as described (32), with the addition of a hot acidic phenol-extraction step after labeling. For hybridization probes, DNA fragments from the HIV-1 genome were cloned, in both orientations, into m13mp19. Clones containing a −18 to +79 fragment were

FIG. 2. Northern blot analysis and Tat complementation of CEM Tat⁻ cell lines. (A) Northern blot of CEM cells infected with the wild-type Tat neo⁺ virus (2-hr exposure) and Tat⁻ CEM cell lines (12-hr exposure). The 10.0-kb, 5.1-kb, and 2.8-kb RNAs correspond to the full-length genomic and singly and doubly spliced mRNAs, respectively. Because the blots were hybridized with an LTR probe, the neo transcript terminating in the 3′ LTR in the wild-type virus and in the sense-orientation Tat⁻ viruses is also seen. Independent clones express slightly variable basal levels of viral mRNAs. (B) Dose-response of Tat protein complementation. Cells (10⁶) were treated with the indicated doses of Tat, and p24 antigen levels were measured after 24 hr.

provided by M. Laspia (Cold Spring Harbor Laboratory, NY). Human γ-actin probes were constructed by cloning the *Bam*HI–*Hin*dIII fragment of pHF1 (33) into m13mp18 and
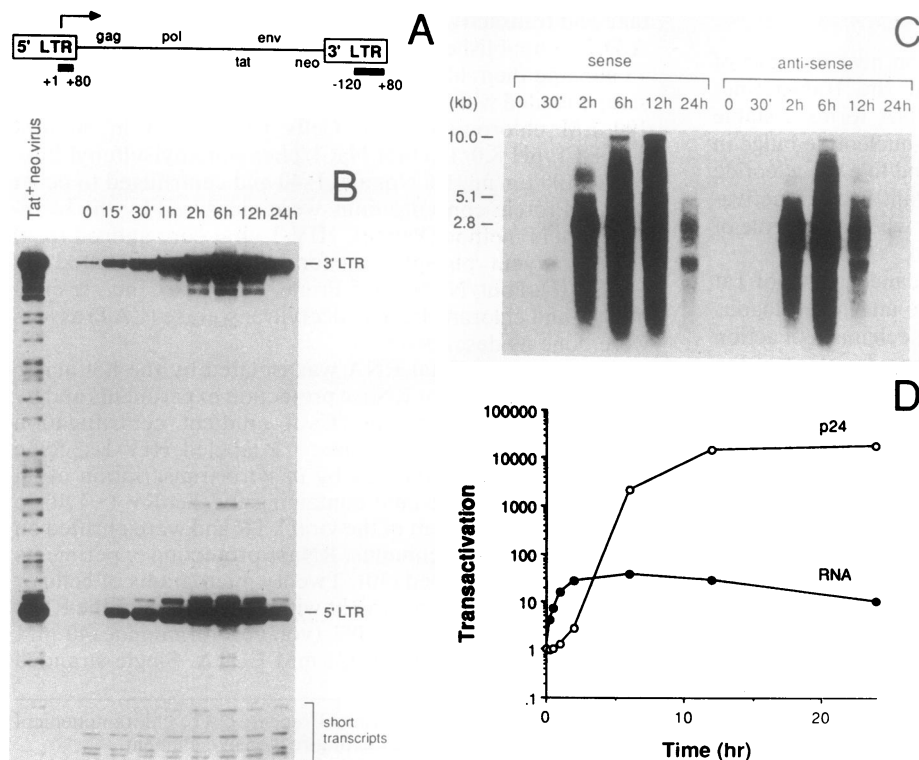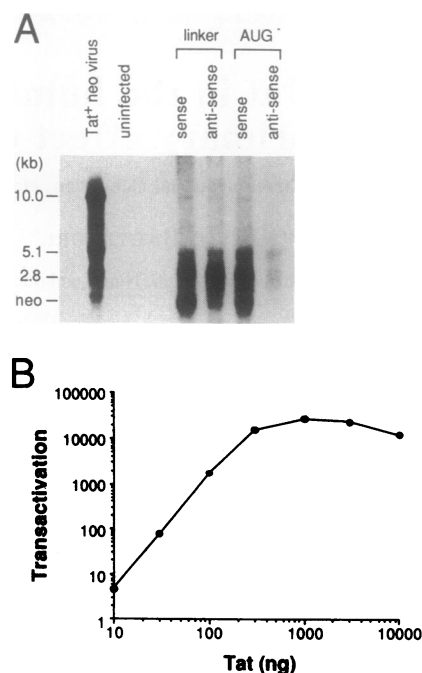

FIG. 3. Time course of Tat complementation in CEM Tat⁻ cells. (A) Map showing the location and sizes of fragments generated by RNase protection using a probe from −120 to +80 of the LTR. (B) Cells were treated with Tat protein, and RNA was prepared and analyzed by RNase protection at the times indicated. The protected fragment from the 5′ LTR extends from the cap site (+1) to +80; the 3′ LTR fragment extends from +8996 to the poly(A) signal at +9195 (equivalent to −120 to +80 in the 5′ LTR). (C) Northern blot analysis of RNA from the same cells. (D) RNA from the RNase protection in *B* was quantitated and compared with p24 antigen levels.

m13mp19. Five micrograms of single-stranded m13 DNAs was UV-crosslinked to nitrocellulose filters, and hybridization, washing, and RNase treatment were as described (32).

RESULTS

Tat-Defective Virus Cell Lines. To obtain cell lines containing faithfully integrated Tat-mutant HIV-1 proviruses, we constructed proviruses that contained a selectable marker (*neo*) in place of a portion of the *nef* gene, which is dispensible for viral replication (Fig. 1*A*). Mutant *tat* genes were introduced into the proviruses (Fig. 1*B*); these are completely inactive for transactivation. Plasmids containing the mutant proviral genomes were transfected into CEM cells, and the cells were treated with Tat protein to complement the *tat* mutations and produce infectious viral particles (see below). The supernatant containing Tat⁻ viruses was used to infect fresh CEM cells, and stable integrants were cloned.

Northern (RNA) blot analysis of several cloned Tat⁻ virus cell lines shows that each has a similar pattern of RNA expression (Fig. 2*A*). Interestingly, the low level of unspliced [10 kilobase (kb)] and singly spliced (5.1 kb) viral mRNAs is similar to that seen with Rev mutants (8) and suggests that little or no Rev is produced in the absence of Tat.

To test how efficiently exogenously added Tat protein complements Tat-defective viruses, Tat⁻ cells were treated with increasing amounts of Tat, and virus production was measured. Levels of p24 increased linearly up to a Tat concentration of 10 μg/ml, resulting in transactivation of >30,000-fold (Fig. 2*B*). The ability of Tat 1–72 to fully complement the Tat deficiency shows that the first exon of Tat is sufficient to support viral replication. Similarly, because both mutations prevent expression of Tev (a *tat–env–rev* fusion protein; refs. 34 and 35), we conclude that Tev is not essential for viral replication.

Time Course of Tat Transactivation in Tat⁻ Virus CEM Cells. To examine the time course of Tat action and assess the possible effects of Tat on transcription and/or translation, we treated a CEM Tat⁻ cell line with Tat protein and monitored expression of viral RNAs by RNase protection and Northern blotting and measured protein expression by determining the intracellular levels of p24. An RNA probe complementary to −120 to +80 of the LTR, which hybridizes to both the 5' and 3' LTRs and yields protected fragments of 80 and 200 nucleotides, respectively (Fig. 3*A*) was used for the RNase protection experiments. After 15 min of Tat treatment, a significant increase in full-length viral mRNAs was apparent by RNase protection (Fig. 3*B*); the maximum was reached by 6 hr. Both the 5' and 3' LTR-derived RNAs increased equally, suggesting that all transcripts initiated after Tat addition were completely elongated. In the absence of Tat (time 0), short transcripts approximately the size of the TAR stem-loop (57 nucleotides) were also seen and did not change significantly during the Tat treatment (Fig. 3*B*). Actinomycin D treatment completely blocked transactivation (data not shown), suggesting that new transcription is required for Tat function and showing that the increase in RNA does not result from mRNA stabilization.

The pattern of RNA expression in the CEM Tat⁻ time course was also followed by Northern analysis to determine which classes of viral mRNAs were being expressed as Tat transactivation proceeded (Fig. 3*C*). As with the RNase protection analysis, mRNA levels increased soon after Tat addition (30 min) and showed maximum expression by 6 hr. The distribution of viral mRNAs shifted from predominantly spliced mRNAs to unspliced and singly spliced mRNAs, indicating that Tat transactivation increased Rev expression. This result strongly supports the idea that HIV-1 gene expression is temporally regulated (36), and that Tat function precedes Rev function.

Levels of intracellular p24 were measured to determine the time course of protein expression. In contrast to the rapid RNA increase, p24 expression did not significantly increase until 2 hr after Tat addition (Fig. 3*D*). Such a lag was expected because p24 expression first requires transcription and translation of Rev before its own mRNA can be translated. The level of p24 plateaued 12 hr after Tat addition and increased 19,000-fold; total RNA levels increased 38-fold (Fig. 3*D*). The difference in transactivation between p24 protein and RNA levels is almost certainly due to a low level of Rev expression in the Tat⁻ viruses. Without Rev, virtually all of the unspliced genomic mRNA, which encodes p24, is located in the nucleus and cannot be translated (37).

Time Course of Tat Transactivation in Tat⁻ Virus HeLa Cells. To directly determine whether the difference in RNA and p24 induction seen in the CEM Tat⁻ virus cells reflects low Rev expression, we measured the time course of transactivation in Tat⁻ virus HeLa cells. In these cells the *neo* gene is under control of the HIV LTR, but, unlike p24 (*gag*), *neo* is located on a spliced mRNA, and so its expression is Rev-independent. This situation allowed us to monitor two reporter proteins simultaneously—one Rev-dependent (p24) and one Rev-independent (neo)—in addition to RNA expression. The pattern of viral RNA expression in the HeLa Tat⁻ cells was virtually identical to that seen in the CEM Tat⁻ cells: RNA levels increased rapidly after Tat addition and reached a maximum after 6 hr, and identical short transcripts
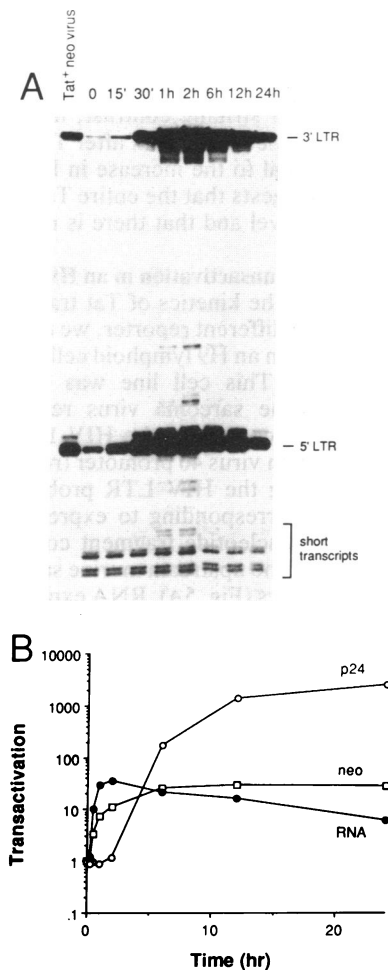

FIG. 4. Time course of Tat complementation in HeLa Tat⁻ cells. (*A*) HeLa cells (10⁷) were scrape-loaded with Tat protein (33), and RNA was analyzed by RNase protection as in Fig. 3. ', min; h, hr. (*B*) Quantitation of RNA, p24 antigen, and neomycin phosphotransferase.
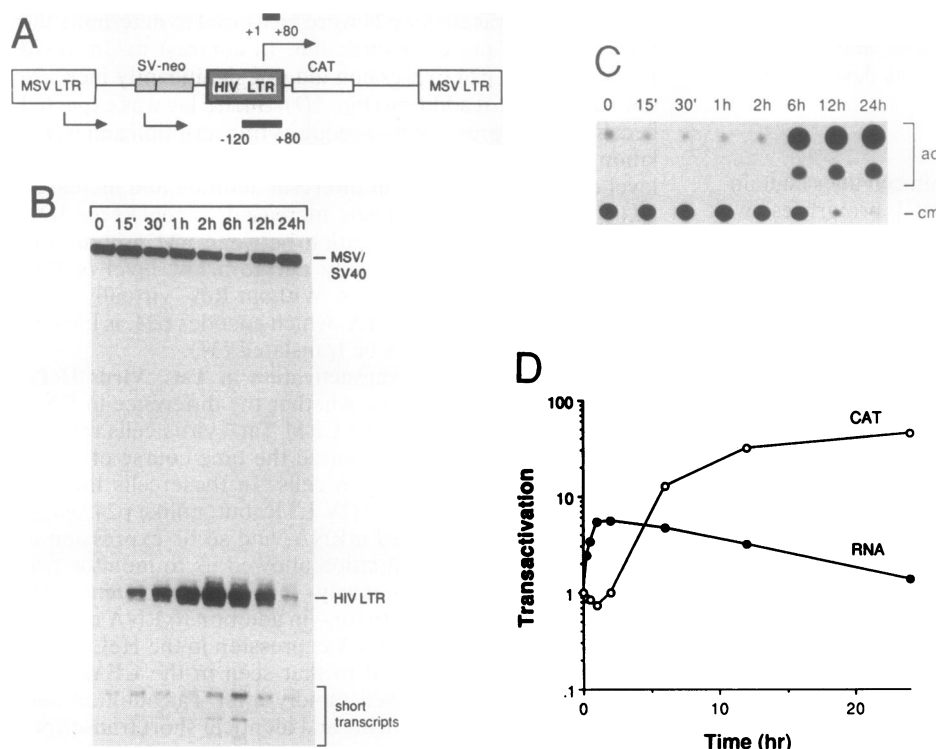
FIG. 5. Time course of Tat comple-
mentation in H9 CAT reporter cells. (*A*)
Map of the murine sarcoma virus (MSV)
retroviral vector used to establish the
H938 cell line (25). (*B*) Cells were treated
with Tat protein, and RNA was analyzed
by RNase protection. (*C*) CAT assay of
the time course. (*D*) Quantitation of RNA
and CAT activity.

were present that did not change in response to Tat (Fig. 4*A*).
The pattern of p24 expression was also similar; a much larger
increase of p24 occurred as compared with RNA (2500-fold
vs. 36-fold; Fig. 4*B*). In striking contrast, neomycin phos-
photransferase II increased by 30 min after Tat addition and
was directly proportional to the increase in RNA (Fig. 4*B*).
This result strongly suggests that the entire Tat effect occurs
at the transcriptional level and that there is no translational
component.

**Time Course of Tat Transactivation in an H9 CAT Reporter
Cell Line.** To compare the kinetics of Tat transactivation in
the Tat⁻ viruses with a different reporter, we measured RNA
and protein expression in an H9 lymphoid cell line containing
HIV LTR CAT (25). This cell line was established by
infection with a murine sarcoma virus retroviral vector
containing CAT under control of the HIV-1 LTR and *neo*
under control of a simian virus 40 promoter (ref. 25; Fig. 5*A*).
RNase protection using the HIV LTR probe gives an 80-
nucleotide fragment corresponding to expression from the
HIV LTR and a 200-nucleotide fragment corresponding to
expression from either the upstream murine sarcoma virus or
simian virus 40 promoters (Fig. 5*A*). RNA expressed from the
HIV LTR increased after Tat addition, showing kinetics
similar to those seen in the CEM and HeLa Tat⁻ virus lines,

whereas transcription from the upstream murine sarcoma
virus or simian virus 40 promoters actually decreased (Fig.
5*B*). Thus, Tat transactivates only the HIV promoter, even
though upstream promoters contribute to basal-level expres-
sion of CAT mRNA.

CAT protein expression increased 47-fold after Tat addi-
tion (Fig. 5 *C* and *D*), while the RNA level increased only
6-fold (Fig. 5*D*). At first glance, this result might suggest a
posttranscriptional effect of Tat; however, it is more likely
that this reflects the relative translation efficiencies of
mRNAs derived from the upstream promoters and from the
HIV promoter rather than a translational effect of Tat. Such
effects on basal-level expression or those resulting from DNA
context, also seen by others (38), may account for reported
differences between RNA and protein transactivation that
have led to suggestions of a posttranscriptional role for Tat.

The Major Effect of Tat Is on Transcriptional Elongation.
Previous studies suggested that Tat may work, at least in
part, by increasing the efficiency of transcriptional elongation
(7, 11). To examine this hypothesis within the virus, we
performed nuclear run-on experiments using probes comple-
mentary to several regions of the full-length genomic tran-
script (Fig. 6*A*). As has been observed (7), there is a strong
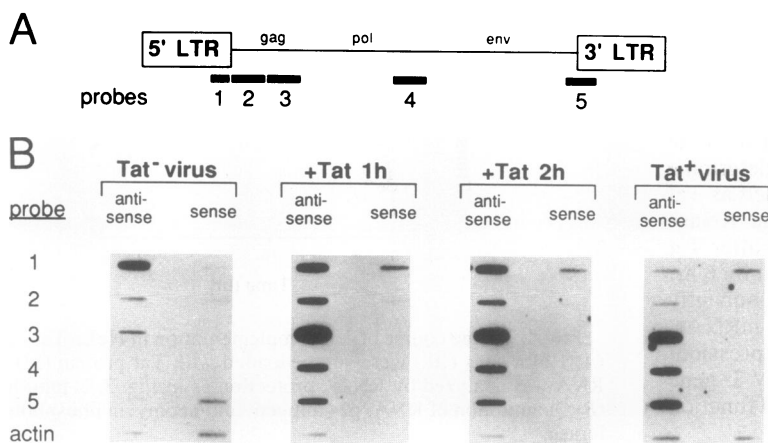polarity to HIV transcription in the absence of Tat, and

FIG. 6. Transcriptional nuclear run-on of
CEM Tat⁻ cells. (*A*) Map showing the location
of the five DNA probes used to determine the
polarity of transcription across the HIV-1 ge-
nome. The probes correspond to sequences
from wild-type HIV-1: probe 1, −18 to +79;
probe 2, +80 to +633; probe 3, +634 to +1260;
probe 4, +4987 to +5610; probe 5, +8624 to
+9098. (*B*) Nuclear run-on assay of CEM Tat⁻
virus cells, Tat⁻ cells treated with Tat for 1 hr
and 2 hr, and wild-type (Tat⁺) virus-infected
cells. After the run-on reaction, the labeled
RNAs were hybridized to anti-sense probes 1–5,
with sense probes 1–5 serving as negative con-
trols. A γ-actin probe was included as an inter-
nal control to normalize for the efficiency of
each run-on reaction.

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polymerase density markedly decreases within a few hundred nucleotides from the 5' end and virtually no transcription is detectable proceeding to the 3' end (Fig. 6B). Significantly, even without Tat, a high level of promoter-proximal transcripts is seen, suggesting a high initiation rate. By 1 hr after Tat addition, distal transcription increases markedly, virtually eliminating the polarity (Fig. 6B). Tat does not seem to affect initiation because the level of the most-5' transcript is unchanged. Because the 5' and 3' ends of the mRNAs increased equally, as observed by RNase protection (Fig. 3B), we conclude that all transcripts initiated in the presence of Tat progress to the 3' end and become polyadenylated.

DISCUSSION

By complementing Tat-defective viruses with the Tat protein, we have shown that (*i*) Tat acts early in the HIV-1 life cycle and precedes Rev function and (*ii*) transactivation results primarily from increased transcriptional elongation. These observations indicate that regulation of HIV-1 gene expression by Tat may be relatively simple but exceedingly powerful. In the absence of Tat, the HIV-1 promoter initiates transcription at a high rate, but nascent transcripts are inefficiently elongated and Rev expression is low. When Tat is added, elongation proceeds efficiently, and large amounts of full-length genomic transcripts are produced. This results in increased Rev expression, which causes accumulation of unspliced mRNAs and expression of viral structural proteins. Thus, Tat regulates two stages of HIV-1 gene expression: Tat acts directly to increase the rate of synthesis of full-length transcripts and acts indirectly by controlling Rev expression.

Tat transactivation within HIV-1 is due primarily to increased efficiency of transcriptional elongation. In the absence of Tat, two classes of RNAs are seen in the Tat⁻ viruses; a low level of full-length genomic RNAs and relatively abundant short RNAs (Fig. 3B). The full-length mRNAs are very stable (they decrease only slightly after 2 hr of actinomycin D treatment; data not shown), suggesting that even this low level represents RNAs accumulated from many prior rounds of initiation. The abundant short RNAs, which appear to be degradation products from incompletely elongated RNAs (39) rather than products of a specific termination site (11), suggest that the rate of initiation is high. When Tat is added, the level of genomic mRNA increases, and virtually all initiated transcripts are efficiently elongated. In a wild-type Tat⁺ virus, elongation proceeds efficiently, continuously generating full-length mRNAs, and no short transcripts accumulate (Fig. 3B). Nuclear run-on experiments support increased elongation; the striking polarity to transcription in the absence of Tat is completely overcome by Tat addition (Fig. 6). Without Tat, the fall-off in transcription is gradual, rather than the result of a specific termination site. Tat does not appear to increase initiation because transcription near the 5' end of the genome is unaffected by Tat. These results support certain aspects of previous studies, showing effects of Tat on transcriptional elongation (7, 11, 13, 38), and suggest that this mechanism may account for the entire effect of Tat within the virus.

Control at the level of transcriptional elongation provides a rapid switch to turn on gene expression. By assembling transcription complexes that lack a factor required for efficient elongation, a promoter can efficiently initiate transcription and be primed for high-level expression as soon as the missing component is supplied. In essence, Tat may be a gene-specific elongation factor, targeted to the HIV-1 promoter by binding to TAR RNA. Once bound to the nascent transcript, Tat might then be positioned to interact directly with the elongation complex to prevent premature termination. Viruses such as HIV-1 may have evolved such a

mechanism to allow for rapid expression after a period of latency. Perhaps cellular genes that show rapid fluctuations in expression during the cell cycle, such as c-*myc*, may be effectively regulated through transcriptional elongation (40), the timing of which is determined by expression of gene-specific elongation factors.

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